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Multiplexed Protein Quantification with Barcoded Hydrogel Microparticles

David C. Appleyard, Stephen C. Chapin, and Patrick S. Doyle*

Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

We demonstrate the use of graphically encoded hydrogel microparticles for the sensitive and high-throughput multiplexed detection of clinically relevant protein panels in complex media. Combining established antibody capture techniques with advances in both microfluidic synthesis and analysis, we detected 1−8 pg/mL amounts of three cytokines (interleuken-2, interleuken-4, and tumor necrosis factor alpha) in single and multiplexed assays without the need for filtration or blocking agents. A range of hydrogel porosities was investigated to ensure rapid diffusion of targets and reagents into the particle as well as to maintain the structural integrity of particles during rinsing procedures and high-velocity microfluidic scanning. Covalent incorporation of capture antibodies using a heterobifunctional poly(ethylene glycol) linker enabled one-step synthesis and functionalization of particles using only small amounts of valuable reagents. In addition to the use of three separate types of single-probe particles, the flexibility of the stop-flow lithography (SFL) method was leveraged to spatially segregate the three probes for the aforementioned target set on an individual encoded particle, thereby demonstrating the feasibility of single-particle diagnostic panels. This study establishes the gel−particle platform as a versatile tool for the efficient quantification of protein targets and significantly advances efforts to extend the advantages of both hydrogel substrates and particle-based arrays to the field of clinical proteomics.

Advances in medical diagnostics and patient-tailored therapy require robust methods for the sensitive and rapid measurement of proteins. Such techniques have the potential to elucidate the active processes that determine disease state, as well as the mechanisms by which drug treatments achieve success. A high-throughput platform for the multiplexed quantification of medically relevant proteins in complex biological samples will narrow the considerable gap that currently separates academic discussions of proteomic analysis from the realities of the clinical setting.[1−3] Although there has been moderate success in monitoring disease state by tracking the expression of a single protein, it is highly likely that focused panels of protein biomarkers will provide the most reliable predictions of therapeutic efficacy and the earliest warnings of disease, even enabling a diagnosis before a patient develops symptoms.[4−6]

Traditionally, protein detection has been carried out with the time- and labor-intensive enzyme linked immunosorbent assay (ELISA), which leverages the variety and specificity of antibodies. A common implementation is the sandwich ELISA, in which a capture antibody raised against the target protein is attached to the surface of a microplate well. A clinical sample is then introduced; the target protein is allowed to bind to the capture antibody, and a second reporter antibody raised against a non-competing epitope of the target protein is added, thus forming a sandwich. The reporter antibody is functionalized for fluorescent or colorimetric signaling. Because of the abundance of validated antibody pairs available for sandwich ELISAs, this detection scheme has been adapted for a number of platforms, including planar and particle arrays.[7,8]

The ability to correlate a reporting event to a specific target species is crucial in developing a multiplexed sandwich assay. With the positional encoding scheme used in planar arrays, capture antibodies are spotted at specific two-dimensional locations, thus providing a high density method to simultaneously measure thousands of targets.[8] However, the fixed design, long incubation times, and low throughput of the format make planar arrays ill-suited for the rapid sample processing and frequent probe-set modifications that are required for diagnostic applications. A particle-based multiplexing array that dopes polystyrene microspheres with combinations of dyes for optical encoding has been developed by Luminex as an alternative format that can provide high-throughput analysis of samples and faster target-binding kinetics. Though adaptable, this system suffers from spectral overlap between encoding and reporting fluorophores, limiting coding capacity to ∼500. Moreover, large intra- and intertrial coefficients of variation (CVs) require large numbers of these particles to be processed to generate high-quality measurements.[9,10]

* To whom correspondence should be addressed. E-mail: pdoyle@mit.edu.

(1) Giljohann, D. A.; Mirkin, C. A. *Nature* **2009**, *462*, 461.
(2) Petricoin, E. F.; Liotta, L. A. *J. Nutr.* **2003**, *133*, 2476S.
(3) Zichi, D.; Eaton, B.; Singer, B.; Gold, L. *Curr. Opin. Chem. Biol.* **2008**, *12*, 78.
(4) De Angelis, G.; Rittenhouse, H. G.; Mikolajczyk, S. D.; Blair Shamel, L.; Semjonow, A. *Rev. Urol.* **2007**, *9*, 113.
(5) Gorelik, E.; Landsittel, D. P.; Marrangoni, A. M.; Modugno, F.; Velikokhatnaya, L.; Winans, M. T.; Bigbee, W. L.; Herberman, R. B.; Lokshin, A. E. *Cancer Epidemiol., Biomarkers Prev.* **2005**, *14*, 981.
(6) Wulfkuhle, J. D.; Liotta, L. A.; Petricoin, E. F. *Nat. Rev. Cancer* **2003**, *3*, 267.
(7) de Jager, W.; te Velthuis, H.; Prakken, B. J.; Kuis, W.; Rijkers, G. T. *Clin. Diagn. Lab. Immunol.* **2003**, *10*, 133.
(8) Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W. P.; Wang, M. J.; Fu, Q.; Shu, Q. P.; Laroche, I.; Zhou, Z. M.; Tchernev, V. T.; Christiansen, J.; Velleca, M.; Kingsmore, S. F. *Nat. Biotechnol.* **2002**, *20*, 359.
(9) Birtwell, S.; Morgan, H. *Integr. Biol. (Cambridge)* **2009**, *1*, 345.

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Several emerging encoded particle technologies are being developed with the intent of outperforming the Luminex system.[9,11] Most of the effort by other groups has been devoted to expanding the number of available codes, but they do not offer an efficient method to rapidly decode and quantify target binding, a deficiency that severely limits their systems' utility in real-world applications. Furthermore, many of the new encoded particles are fabricated from standard photoresist materials such as SU8 that foul easily and are not well-adapted to bioassays. This leads to poor sensitivity and large amounts of variability (e.g., 5 nM or 1 μg/mL sensitivity and CV ~50% for IgG detection).[9,12] High CVs are also encountered on "barcoded chips" due to limitations in the manufacturing process.[13] Besides the Luminex platform, metallic barcoded rods are the most mature technology in this field, but few protein immunoassays have been developed thus far for the system, and the demonstrated limits of detection (~100 pg/mL for cytokines)[14] are at least 2 orders of magnitude higher than ELISA. A new approach to high sensitivity quantification, the digital ELISA, can detect down to 10 fg/mL for tumor necrosis factor alpha, TNFα, yet does not offer high-throughput scanning nor multiplexing.[15] The failure of these systems to provide a versatile approach to multiplexed protein quantification has slowed the development of clinical proteomics. The ideal particle for protein assays would feature a nonfouling and biologically inert substrate, a robust and inexpensive system for rapid synthesis and analysis, a large coding library, and a sensitivity of detection comparable to that of the leading technologies in the field.

The bulk immobilization of capture molecules within hydrated gel matrixes is fundamentally different from the spotting of probe onto the solid substrates that are used in planar microarrays and current particle-based systems. Hydrogels have previously been used in combination with planar array formats to provide a permeable 3-D scaffold for the bulk immobilization of capture antibody, thereby augmenting the loading capacity, improving the binding kinetics, and reducing steric issues caused by rigid attachment.[16−18] Encapsulation of enzymes and measurement of a bienzymatic reaction have been demonstrated using suspension arrays of hydrogel particles.[19] The bioinert nature of poly(ethylene glycol) (PEG) offers the added advantage of reduced nonspecific binding, thereby eliminating the requirement for preanalysis purification steps.[20]

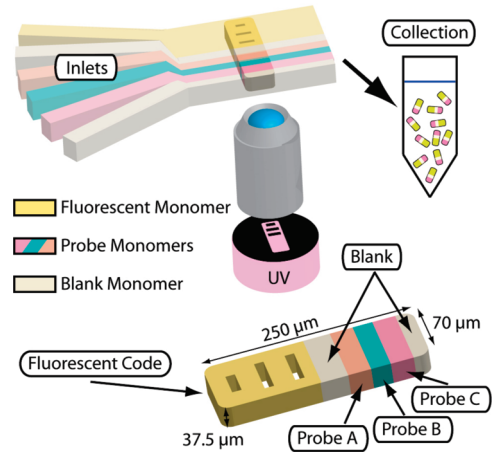

Figure 1. Schematic of the SFL system for synthesis of a three-probe hydrogel particle formed in a microfluidic device with six pressure-driven inlet streams. The streams are combined into a single channel where the individual stream widths can be adjusted by modulating the inlet pressure. UV exposure causes polymerization of the streams into a particle shape defined by a transparency mask. A collection tube is attached to the end of the microfluidic channel, and particles are gathered so they can be washed before storage or use. A variety of chemistries can be loaded into each inlet stream. For the three probe particle, a fluorescent monomer (yellow) forms the code and a blank monomer stream without fluorophore or probe (gray) is used to separate the code and detection regions. Three probe monomer streams with unique capture antibodes are used to allow multiplexed detection (orange, blue, and pink). Finally, an additional blank stream (gray) is used to cap the end of the particle. The number of probe streams can be altered to make single-probe or multiple-probe particles, depending on assay requirements.

The use of barcoded hydrogel microparticles to quantify panels of protein targets represents a significant paradigm shift in multiplexed immunoassays. When the detection advantages of gel scaffolds are combined with the operational efficiencies of particle-based arrays, the platform described herein represents a powerful new tool for rapid and sensitive protein quantification that can easily incorporate commercially available antibody pairs. The microfluidic stop-flow lithography (SFL) technique is used to simultaneously synthesize, encode, and functionalize gel particles (>10^4/h) with a fluorescent barcoded region consisting of unpolymerized holes and a spatially segregated multiprobe region embedded with capture antibodies (Figure 1).[21] This arrangement allows a single fluorophore to be used, eliminating restrictions from spectral overlap and allowing for the use of a simple and inexpensive detection apparatus. The code is composed of an expandable series of five bits, each bit having a value of 0, 1, 2, or 3. One bit is fixed to provide particle orientation information, thereby providing 192 unique codes which can easily be augmented to >10^5 by adding more bits. A large code library enables multiplexed detection of numerous targets and the ability to pool samples from a large patient population into a single analysis. Because the particles are composed of bioinert

(10) Krishhan, V. V.; Khan, I. H.; Luciw, P. A. *Crit. Rev. Biotechnol.* **2009**, *29*, 29.
(11) Finkel, N. H.; Lou, X.; Wang, C.; He, L. *Anal. Chem.* **2004**, *76*, 352A.
(12) Broder, G. R.; Ranasinghe, R. T.; She, J. K.; Banu, S.; Birtwell, S. W.; Cavalli, G.; Galitonov, G. S.; Holmes, D.; Martins, H. F.; Macdonald, K. F.; Neylon, C.; Zheludev, N.; Roach, P. L.; Morgan, H. *Anal. Chem.* **2008**, *80*, 1902.
(13) Fan, R.; Vermesh, O.; Srivastava, A.; Yen, B. K.; Qin, L.; Ahmad, H.; Kwong, G. A.; Liu, C. C.; Gould, J.; Hood, L.; Heath, J. R. *Nat. Biotechnol.* **2008**, *26*, 1373.
(14) Brunker, S. E.; Cederquist, K. B.; Keating, C. D. *Nanomedicine (London)* **2007**, *2*, 695.
(15) Rissin, D. M.; Kan, C. W.; Campbell, T. G.; Howes, S. C.; Fournier, D. R.; Song, L.; Piech, T.; Patel, P. P.; Chang, L.; Rivnak, A. J.; Ferrell, E. P.; Randall, J. D.; Provuncher, G. K.; Walt, D. R.; Duffy, D. C. *Nat. Biotechnol.* **2010**, *28*, 595.
(16) Moorthy, J.; Burgess, R.; Yethiraj, A.; Beebe, D. *Anal. Chem.* **2007**, *79*, 5322.
(17) Rubina, A. Y.; Kolchinsky, A.; Makarov, A. A.; Zasedatelev, A. S. *Proteomics* **2008**, *8*, 817.
(18) Zubtsov, D. A.; Savvateeva, E. N.; Rubina, A. Y.; Pan'kov, S. V.; Konovalova, E. V.; Moiseeva, O. V.; Chechetkin, V. R.; Zasedatelev, A. S. *Anal. Biochem.* **2007**, *368*, 205.
(19) Lee, W.; Choi, D.; Kim, J. H.; Koh, W. G. *Biomed. Microdevices* **2008**, *10*, 813.
(20) Wan, J.; Thomas, M. S.; Guthrie, S.; Vullev, V. I. *Ann. Biomed. Eng.* **2009**, *37*, 1190.
(21) Chapin, S. C.; Appleyard, D. C.; Pregibon, D. C.; Doyle, P. S. *Angew. Chem.* **2010**, in press.

PEG, they are nonfouling and have minimal nonspecific binding, making them favorable for assays of targets in complex biological samples that may contain a thousand-fold to million-fold excess of background protein species.

Adjusting the ratio of the cross-linking monomer to an inert porogen provides the ability to tune hydrogel porosity and permeability. Since the number of probe regions on the particle can easily be modified by adding or removing stream inlets on the microfluidic device, multiplexed detection can be established either on a single particle or through the combination of multiple particles.

In this paper, we demonstrate the ability to adapt traditional sandwich antibody detection to a multiplexed barcoded hydrogel assay for three cytokines, two from the CD132 family, interleukin-2 (IL-2) and interleukin-4 (IL-4), as well as tumor necrosis factor alpha (TNFα).[4] This target panel is of high clinical value because cytokine signaling initiates the immune response and is responsible for maintenance of the immune system memory. The IL-2 family is associated with the development of adaptive immunity. IL-4 is directly involved in T-helper type 2 cell differentiation, and in combination with IL-2, it has been shown to regulate T-cell fate.[22,23] TNFα is linked to the inflammatory response, is present in septic shock, and can even act as a cancer therapy.[24]

EXPERIMENTAL SECTION

Assay Overview. The protein detection assay occurs in three primary steps, which are detailed explicitly in the following sections: Particle Synthesis, Protein Detection, and Particle Scanning. Briefly, particles with capture antibody probes were synthesized in bulk and were stored, up to three months, before use. During protein detection, approximately 50 particles were combined with a sample containing the target protein and incubated for 2 h. The particles were subsequently washed and mixed with biotinylated reporter antibodies and incubated for 1 h. Another wash step removed unbound reporter antibody prior to an addition of streptavidin-conjugated fluorophore. After a 30 min incubation, excess fluorophore was removed with a final wash. Particles were immediately resuspended in a scanning buffer and loaded onto a microfluidic device for rapid readout of the barcode and target level.

Particle Synthesis. Capture antibodies were functionalized by incubating 10 μL of 25 μg/μL antibody with 2.5 μL of a 60 μg/μL solution of 2 kDa heterobifunctional PEG linker (ACRL-PEG-SCM-2000, Laysan Bio) for 3 h at 25 °C while agitating at 60 rpm. This mixture was combined in a 1:9 ratio with a 20% (v/v) poly(ethylene glycol) diacrylate (PEG−DA) monomer mixture (20% PEG−DA (MW 700 g/mol), 40% PEG (MW 200 g/mol), 5% Darocur 1173, 35% 3× Tris−EDTA, pH 8.0 (TE)) to form the probe prepolymer. Code prepolymer was formed by combining rhodamine acrylate at 0.06 mg/mL in 1× TE at 1:9 with a 35% PEG−DA monomer mixture (35% PEG−DA, 20% PEG, 5% Darocur 1173, 40% 3× TE). A 1:5 mixture of food coloring in 1× TE was added at 1:9 with the 35% PEG−DA monomer solution to form the blank prepolymer.

Particles were synthesized using methods explicitly detailed in Pregibon et al.[25] In brief, the prepolymer mixtures were loaded into separate modified pipette tips and then injected into a poly(dimethylsiloxane) (PDMS) microfluidic device. A pressure distribution system was used to generate laminar, coflowing streams in a rectangular region of the device of width 270 μm and height 40 μm, resulting in a polymerized particle that has a height of 37.5 μm (Figure 1A). The width of each prepolymer stream was precisely controlled by adjusting inlet pressure via relief valves.[25] Probe regions were adjusted to be 35 μm in length in the single- and multi-probe particles. This length was set to balance sensitivity with scanning accuracy. The region had to be long enough to be accurately quantified during scanning by the photomultiplier tube (PMT), yet short enough to concentrate signal from dilute samples. Buffer streams were placed between the code and probe as well as at the end of the particle to limit edge effects on target diffusion, prevent accidental rhodamine incorporation in the probe strip, and reduce the probe width for increased sensitivity. Polymerization was initiated through selective UV exposure of the monomer streams using a negative mask of the barcoded particle (Figure 1). Computer automation of pressure valves and illumination shutter enabled production of 300 particles per minute using a five-particle mask. Each synthesis cycle used a flow period of 500 ms to establish streams, a stop time of 300 ms to halt flow, a UV exposure time of 100 ms to polymerize particles with a Lumen 200 (Prior Scientific, 75% setting) with a 0.05 neutral density filter inline, and a hold period of 150 ms for completion of polymerization.

Antibody Reagents. IL-2: capture (R&D Systems MAB602), reporter (R&D Systems BAF202), protein (R&D Systems 202-IL). TNFα: capture (R&D Systems MAB610), reporter (R&D systems BAF210), protein (R&D Systems 210-TA). IL-4: capture (R&D Systems MAB604), reporter (R&D Systems BAF204), protein (R&D Systems 204-IL). Before use, antibodies were resuspended in 1× phosphate buffered saline (PBS; Cellgro) with 0.1% bovine serum albumin (BSA) to either 25 μg/μL (capture antibodies) or 500 ng/μL (reporter antibodies). Target proteins were resuspended according to manufacturer's specifications.

Incorporation Efficiency. Polyclonal antithrombin antibodies (Haematologic Technologies PAHT-S) were functionalized by incubating 10 μL of 100 μM antibody with 2.5 μL of a 60 μg/μL solution of a 2 kDa heterobifunctional PEG linker (ACRL-PEG-SCM-2000, Laysan Bio) for 3 h at 25 °C while agitating at 60 rpm. A 5 μL aliquot of 7.67 μM Alexa-532 labeled thrombin was combined with the functionalized antibodies and incubated an additional hour. The antibody/thrombin mixture was combined at a 1:9 ratio with a 20% PEG−DA monomer mixture. The prepolymer mixture was loaded into a microfluidic channel (500 μm wide by 37.5 μm high), and a cylinder-shaped plug 150 μm in diameter was polymerized. A fluorescence image of the particle was taken, followed by a buffer exchange (100× channel volume over 10 min) to wash away unattached antibodies. A second image was taken, and the relative fluorescent intensities of the plug were used to estimate the percentage of antibody remaining covalently integrated into the network.

Protein Detection. For single-plex calibration, 50 particles were added to each filter plate well (Millipore MBVN1210)

(22) Sokol, C. L.; Barton, G. M.; Farr, A. G.; Medzhitov, R. *Nat. Immunol.* **2008**, *9*, 310.
(23) Yates, J.; Rovis, F.; Mitchell, P.; Afzali, B.; Tsang, J. Y.; Garin, M.; Lechler, R. I.; Lombardi, G.; Garden, O. A. *Int. Immunol.* **2007**, *19*, 785.
(24) Kindt, T. J.; Goldsby, R. A.; Osborne, B. A.; Kuby, J. *Kuby immunology*, 6th ed.; W.H. Freeman: New York, 2007.
(25) Pregibon, D.; Doyle, P. *Anal. Chem.* **2009**, *81*, 873.

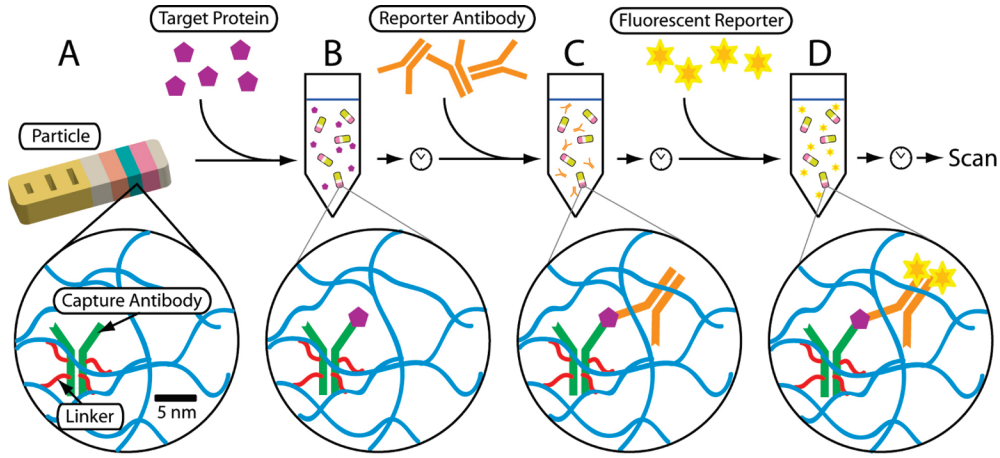

Figure 2. Overview of the protein detection assay, showing an expanded view of the polymer network. (A) Premade particles containing single or multiple capture antibody probes covalently linked into the polymer network are removed from storage. (B) Particles are combined with sample containing the target protein (purple pentagon) incubated, and then washed to remove unbound target and any other contaminants from the sample. (C) Biotinylated reporter antibody (orange) is added to the particles and incubated to form a sandwich with the protein target. A wash step is then performed to remove unbound reporter antibody. (D) Streptavidin phycoerythrin (SAPE, yellow stars) is bound to the complexes after incubation. A final wash step removes unbound SAPE.

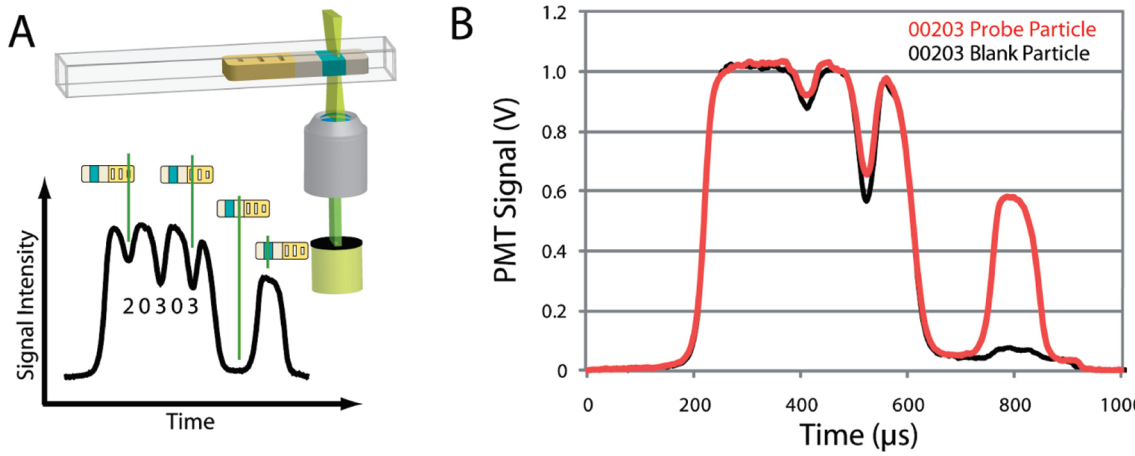

Figure 3. (A) Schematic of the scanning system used on a single probe barcoded particle. Actual scan data demonstrates how a particle is "read" providing the barcode identification and target levels. (B) Actual PMT data for a single-probe particle capturing IL-2 (code "00203"). Red line is the signal intensity for a 60 pg/mL sample; black line is a blank control particle incubated with no target present.

containing the target protein in FBST (1× fetal bovine serum, Invitrogen F2442, with 0.05% Tween-20) and incubated at room temperature for 2 h under agitation (600 rpm; Figure 2). Wells were washed with three 200 μL volumes of PBST (1× PBS with 0.05% Tween-20). Reporter antibody was added at 5 ng/μL in PBST to the wells to give a final volume of 50 μL, and the mixture was then incubated for 1 h at room temperature under agitation. The wells were washed with three 200 μL volumes of PBST. Reporter antibodies bound to protein targets were fluorescently labeled by adding 50 μL of 4 ng/μL streptavidin−phycoerythrin (SAPE) in PBST to each well and incubating for 30 min at room temperature while shaking. Particles received a final wash with three 200 μL volumes of PBST and were resuspended in 50 μL of PTET (5× TE with 25% (v/v) PEG 400 and 0.05% Tween) for scanning. For multiplexed detection using the interplex format with three single-probe particles with three unique barcodes, the same spike-in assay was used with 50 of each particle type added to every well along with permutations of the target proteins. The intraplex assay, with all three probes on one single particle with only one code, required only 50 particles for each well and reporter antibody concentrations at 1.25 ng/μL.

Particle Imaging. Static images were taken with a cooled interline CCD (Andor Clara) using a 0.05 s exposure time and illumination with a metal arc light source (Lumen 200). Images were combined and contrast adjusted using ImageJ (NIH).

Particle Scanning. Particles were scanned using a flow focusing microfluidic device as described in detail by Chapin et. al[21](Figure 3). Forty microliters of particles suspended in PTET were loaded onto the device, flow aligned, and scanned past a thin line illumination from a 100 mW 532 nm laser (Dragon Lasers) integrated into an inverted microscope (Zeiss Axio Observer A1) with a 20× 0.5 NA objective. Particles were scanned within an hour of resuspension in PTET; there was no detectable change in reporter fluorescence during this period. Fluorescence signal was collected with a PMT (Hamamatsu H7422−40), amplified using in-house designed circuitry, digitized, and stored on a PC using a National Instruments USB-6251 board at 600 kHz

sample rate. Scan analysis (decoding and bound-target quantification) was completed using custom MATLAB routines.

RESULTS AND DISCUSSION

Particle Design and Coupling Efficiency. Exploring a range of porosities by varying the ratio of reactive PEG−DA to PEG porogen in the prepolymer mixture allowed determination of a composition which would allow for quick diffusion of target proteins, antibodies, and SAPE while also maintaining structural integrity for rinsing and scanning procedures. Diffusion experiments were undertaken to estimate the penetration time for 150 kDa IgG molecules as a function of diacrylate to porogen ratio (see Supporting Information). Penetration occurred in 65 s at the lowest diacrylate concentration used, 15%; however, the reduced rigidity of the particles produced less consistent scan results, due to particle compression in the scanner and particle breakage during filtration. Increasing the diacrylate concentration to 20% in the probe region improved stability with fewer than 4% of the particles breaking during filtration. The penetration time was only increased by 34% to almost 100 s using the 20% composition, suggesting a polymer network which would allow target diffusion. Prior work with 20% hydrogel particles used a 30 min incubation with the 300 kDa SAPE to label captured targets.[25] At less than 30 kDa, the target proteins were expected to also fully penetrate the probe region in that time, but a 2 h incubation was used to ensure that equilibrium between dispersed target and immobilized probe was achieved in our trials.

Covalent linkage of the capture antibody to the hydrogel network allowed single-step synthesis of encoded particles and enabled spatial segregation of different capture antibodies on one particle. First, primary amines on the antibody were PEGylated using the *N*-hydroxysuccinimide (NHS) terminator on the heterobifunctional PEG, and then, the acrylate moiety on the other end was incorporated into the polymer network during particle synthesis. Conjugation into the hydrogel may also have occurred through direct reaction of the PEG diacrylate monomer with sulfhydryl groups on the antibody.[26] The specific mechanism of incorporation was not determined; however, synthesis without prior PEGylation of the antibody was hindered by solubility issues. Estimation of incorporation efficiency through the measurement of fluorescent intensity retained after polymerization suggested that 26 ± 3% of the antibody in the prepolymer mixture was retained in the final particle. The improved incorporation efficiency over that of DNA probes (10%) may be attributed to the multiple sites for PEGylation or sulfhydryl reaction increasing the probability of covalent linkage.[25] The improved incorporation efficiency and the microfluidic synthesis approach provided a cost-effective method for synthesis of particles using a small volume of antibody. Five hundred micrograms of capture antibody would produce approximately 60 000 particles, enough for over 1200 assays.

Detection Capabilities. Detection performance of the sandwich assay was examined by generating standard calibration curves for each of the three proteins. To approximate the background protein content expected in clinical samples, each of the three targets was separately spiked into fetal bovine serum (FBS; Figure 4). No additional blocking proteins or filtration steps were necessary, thereby reducing assay complexity and eliminating potential sources of contamination.[27] Conservative incubation times were used to maximize the limit of detection, LOD, by approaching equilibrium binding conditions. Signal-to-noise ratios (SNRs) were calculated to determine the limit of detection, target-level CV, and the dynamic range for each of the targets (Table 1). The limit of detection was defined as the point where a fit to the SNR as a function of target concentration reached three. Intra-run variations in signal were used to calculate the CV. Hydrogel particles consistently exceeded the sensitivity of planar arrays, which required additional signal amplification to reach the detection limits stated in Table 1.[8] Additional filtration was necessary for bead arrays to match the hydrogel detection limit.

The dissociation constant, conjugation of linker, and loading density of the antibodies play a direct role in determining the limit

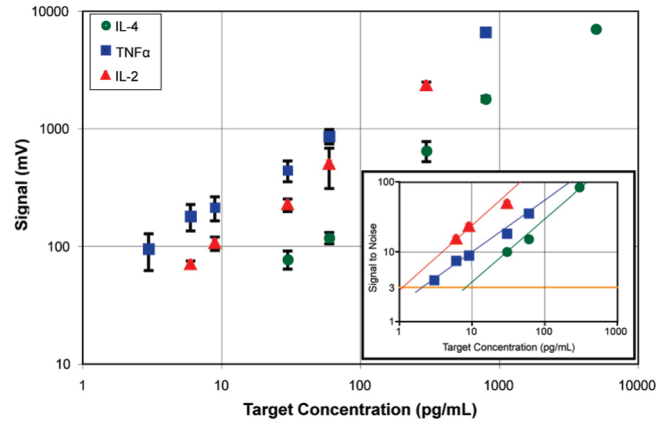

Figure 4. Standard curves for IL-2, TNF-alpha, and IL-4 in FBS. Each point represents the average of 5 particles (IL-2), 20 particles (TNF-alpha), and 13 particles (IL-4). Inset: Signal to noise (S/N) plot, limit of detection was defined as the point where the extrapolated line intersected a S/N of 3 (orange line).

Table 1. Detection Limits, Ranges, and Coefficients of Variation for Bead Arrays, Planar Arrays, Sandwich ELISA, and Hydrogel Particles[a]

target	assay method	LOD (pg/mL)	CV (%)	range ($\log_{10}$)
IL-2	bead array[7,27,30]	1.8[D]-8	9	3.5
	planar array[8]	7	NS	3
	ELISA[A]	1.7	3.4	3
	hydrogel particle	1.1	8.7	3
IL-4	bead array[7,27,30]	1.2[D]-8	8.9	3.5
	planar array[8]	10	35	3
	ELISA[B]	1.6	9.4	3
	hydrogel particle	8.4	11.4	3
TNFα	bead array[7,27,30]	1.2[D]-8	7.4	3.5
	planar array[8]	10	7	3
	ELISA[C]	2.2−4.4	NS	3
	hydrogel particle	2.1	8	3

[a] LOD: limit of detection, defined as the point at which background corrected signal divided by the standard deviation of the blank sample equaled three; CV, intrarun coefficient of variation defined as the standard deviation of the signal divided by the mean of the signal averaged over all concentrations tested in the standard curve in Figure 2. A: Product information R&D systems QuantiGlo IL-2 Immunoassay (Q2000B); B: Product information R&D systems QuantiGlo IL-4 Immunoassay (Q4000); C: Product information R&D systems QuantiGlo TNFαImmunoassay (QTA00B); D: After filtration of original samples; NS: not specified.

(26) Salinas, C. N.; Anseth, K. S. *Macromolecules* **2008**, *41*, 6019.

(27) de Jager, W.; Prakken, B. J.; Bijlsma, J. W. J.; Kuis, W.; Rijkers, G. T. *J. Immunol. Methods* **2005**, *300*, 124.

of detection. Higher affinity antibodies increase the amount of target bound at equilibrium, thus improving signal strength. Similarly, linker conjugation, which alters or limits access to the binding pocket, can directly impact affinity. The capture antibody density plays a direct role in determining the limit of detection. A higher density within the particle will drive equilibrium away from free target in solution and toward captured complexes, increasing the number of bound fluorophores in the probe region. In low concentration samples, this improved sequestering of target results in enhanced signal and thus lowered limit of detection. Other methods for incorporating the capture antibodies at higher density as well as alternate capture molecules, like aptamers, are potential avenues for improved detection.[28] Though the CV in hydrogel and bead arrays are similar, the Luminex system requires many more particles to be evaluated to find a suitable population for quantification. The graphical barcode and microfluidic scanner enables quantification using fewer than 25 hydrogel particles whereas the spectral coding of bead arrays may require hundreds or thousands of particles.[29] The signal response per unit bound target is greater for proteins than for miRNA or DNA systems previously evaluated using hydrogel particles. This can be attributed to the reporter antibody having multiple binding sites for fluorophores, as opposed to the single site of a nucleic acid. While this improves the limit of detection, it also reduces the dynamic range as the PMT detector saturates at high target concentrations. It should be noted that this is a limitation associated with the particular detector used in our setup and not with the saturation of the immobilized probes. The selection of a different PMT with an expanded range would correspondingly augment the detection capacity of the platform.

Multiplexed Detection. Multiplexed detection is essential for effectively monitoring complex systems in clinical diagnostics or research applications. The SFL method enables the spatial segregation of multiple probes on a single particle, yielding convenient colocalization of common target groups. Graphical barcoding enables the use of multiple particle types in a single sample without the limitations imposed by spectral overlap of encoding fluorophores with target quantification fluorophores, thereby enabling rapid probe-set modification for the investigation of a wide variety of target panels. In complex samples, streamlined identification of barcode and quantification of target abundance is obtained using the microfluidic flow-focusing device. The current design can scan the contents of a 50 μL sample well in 30 s, and serial scanning of a standard curve can be completed in under an hour even with manual sample loading of the PDMS device. In future applications, this throughput can be expanded by one to two orders of magnitude with the implementation of an automated liquid handling system.

Cross-reactivity of reagents is one of the primary challenges in developing a multiplexed protein assay.[3] Therefore, it is vital that any new protein quantification platform be capable of integrating reagents already shown to be compatible. This significantly reduces barriers to assay development and enhances the utility of the emerging platform for immediate application. Multiple research groups and commercial sources have assembled validated antibody panels which could be seamlessly paired with our hydrogel technology. We were able to demonstrate simultaneous detection of the three cytokine targets using three uniquely barcoded particles with a single probe strip. All possible combinations of the three cytokines were examined to isolate and identify potential sources of cross-reactivity (Table 2). Microfluidic scanning and analysis of the particles provided quick identification of target type and level. Recovery of the FBS spike-ins was within 20% of predicted values from the calibration curve (Table 2). Most importantly, no significant source of cross reactivity between antibody pairs was encountered.

Table 2. Multiplex Detection of Three Cytokines Using the Interplex, Three Particle System[a]

target			signal (mV)[A]		
IL-4	IL-2	TNFα	IL-4	IL-2	TNFα
+	+	+	927 ± 98	1271 ± 96	1471 ± 145
+	+	−	750 ± 161	922 ± 143	128 ± 57
+	−	+	666 ± 33	76 ± 15	1055 ± 77
−	+	+	−88 ± 64	1243 ± 129	1382 ± 46
−	−	+	35 ± 40	51 ± 42	1340 ± 122
−	+	−	−144 ± 87	1105 ± 41	45 ± 11
+	−	−	850 ± 128	13 ± 7	70 ± 109
−	−	−	0 ± 92	0 ± 8	0 ± 17
recovery (%)[B]			101.7 ± 15.9	118.0 ± 16.5	93.1 ± 16.7

[a] A: Signals were background corrected by subtraction of the control particle (−,−,−), resulting in small negative signals for some conditions where targets were absent. Presence of target (+) correctly corresponds to detected signal. Data taken from five particles for each target under each incubation condition. Negative values are the result of incubations in which the target amount was below the system limit of detection and reflect the noise that dominates the system in the case where there is no detectable signal from the target. B: Recovery for spiked samples calculated as scanner measured concentration using standard curves in Figure 2 divided by the actual spike in concentration. Values are mean ± SD for the four multiplex samples with target present. TNFα spiked at 0.4 ng/mL, IL-2 spiked at 0.12 ng/mL, and IL-4 spiked at 0.11 ng/mL.

A major advantage of our hydrogel design is the ability to spatially address probe location in combination with barcode identification. We created particles with all three probes proximally located on a single hydrogel particle. This "intraplexing" reduces the number of particles necessary for an assay, allows the creation of a single code particle for a specific marker set, and accelerates the reading throughput of the flow cytometer, while also extending the available code base to allow for higher degrees of multiplexing and patient sample pooling. Under the same conditions as the interplex, we were able to detect all three targets on a single barcoded particle (Figure 5). As in the earlier trial, the limited cross-reactivity in this intraplex assay, even at relatively high concentrations, indicates the compatibility of the hydrogel system with conventional antibody pairs and panels. Expanding the number of probes on the particle only requires the simple addition of extra monomer streams producing up to a maximum of eight probe regions on a 250 μm particle with limited barcoding. The simple scalability of intraplex particles results in efficient use of particle space in assays with limited sample volumes.

Future Directions. Experimentation with additional antibody pairs used in ELISA and bead-based assays will continue to expand the available targets for multiplexed detection. Further modeling of LOD as a function of antibody loading will allow for a more tailored detection range and potentially enhanced sensitivity. Using a microfluidic device capable of holding particles while allowing

(28) Cho, E. J.; Collett, J. R.; Szafranska, A. E.; Ellington, A. D. *Anal. Chim. Acta* **2006**, *564*, 82.
(29) Peck, D.; Crawford, E. D.; Ross, K. N.; Stegmaier, K.; Golub, T. R.; Lamb, J. *Genome Biol.* **2006**, *7* (7): R61.

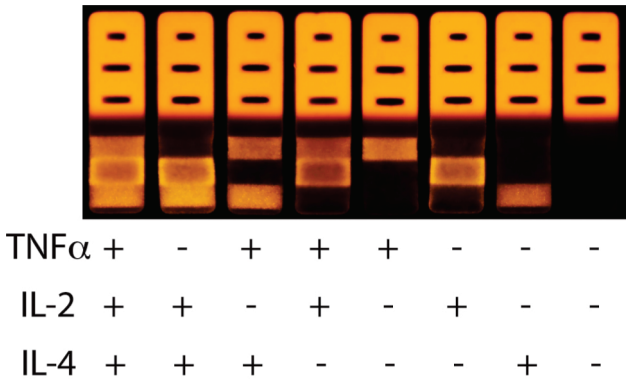

Figure 5. Multiplexed detection on multiprobe particles ("intraplex"). From the top, probe strips are TNFα, IL-2, and IL-4. TNFα spiked at 0.4 ng/mL, IL-2 spiked at 0.12 ng/mL, and IL-4 spiked at 0.11 ng/mL.

for buffer exchange, like that described in Zhang et al., provides the potential for integrating protein detection and scanning steps onto a single device for improvements in throughput and reagent consumption.[31]

CONCLUSIONS

Barcoded hydrogel particles combined with the microfluidic scanning system provide a high-throughput platform for multiplexed quantification of protein abundance and need not be limited to DNA and RNA detection. The ability to covalently incorporate antibodies into the polymer matrix allows for the creation of single- and multi-probe particles capable of measuring clinically relevant amounts of proteins with limits of optical detection, coefficients of variation, and dynamic ranges comparable to leading methods capable of being implemented in a clinical environment. On-particle multiplexing demonstrates the unique capacity to spatially and geometrically encode particles without concern for spectral overlap, while allowing for high-speed reading with a microfluidic flow-through scanner. We also believe this concept will allow for the design of new assays. Use of a bioinert PEG hydrogel reduces background in complex samples and eliminates the need for blocking proteins or prefiltration of samples. The successful combination of multiplexed protein quantification with the versatile nature of SFL provides a flexible platform for clinical diagnostics as well as tailored research applications.

(30) Lagrelius, M.; Jones, P.; Franck, K.; Gaines, H. *Cytokine* **2006**, *33*, 156.
(31) Zhang, H.; DeConinck, A. J.; Slimmer, S. C.; Doyle, P. S.; Lewis, J. A.; Nuzzo, R. G. *Chem.: Eur. J.* **2010**, in press.

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SUPPORTING INFORMATION AVAILABLE

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